Available online at www.sciencedirect.com
SCIENCE DIRECT
Toxicology 222 (2006) 165–174
TOXICOLOGY
www.elsevier.com/locate/toxicol

The role of tumor necrosis factor α and the peroxisome proliferator-activated receptor α in modulating the effects of fumonisin in mouse liver

Kenneth A. Voss [a], Ronald Riley [a], Corrie Dunn [b], J. Christopher Corton [b,*]

[a] *USDA, 950 College Station Road, P.O. Box 5677, Athens, GA 30604-5677, United States*
[b] *CIIT Centers for Health Research, 6 Davis Drive, Research Triangle Park, NC 27709, United States*

Received 3 November 2005; received in revised form 13 February 2006; accepted 16 February 2006
Available online 28 February 2006

Abstract

Fumonisins are mycotoxins that are produced by *Fusarium verticillioides* found in corn and corn-based foods, and are suspected human esophageal carcinogens. Exposure of rodents to fumonisin B_1 causes hepatotoxicity and results in alterations in the balance between cell proliferation and apoptosis in the liver. As the cytokine tumor necrosis factor α (TNFα) and the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) also modulate hepatocyte proliferation and apoptosis, we tested the hypothesis that fumonisin-induced hepatotoxicity in the liver is modulated by these factors. We examined the effects of dietary exposure to a fumonisin-containing culture material (CM) of the fungus *F. verticillioides* for 8 days or 5 weeks in the livers of mice lacking either TNFα or PPARα. Compared to wild-type mice TNFα-null mice exhibited increased hepatocyte proliferation and apoptosis. In contrast, PPARα-null and wild-type mice were found to exhibit similar patterns of hepatocyte apoptosis and proliferation when fed the CM diet. Overall, these findings provide evidence that TNFα, but not PPARα, plays a role in modulating fumonisin-induced hepatotoxicity in mice.
Published by Elsevier Ireland Ltd.

Keywords: Peroxisome proliferator-activated receptor α; Fumonisin; Tumor necrosis factor α; Apoptosis; Hepatocyte proliferation; Sphingolipids

1. Introduction

Fumonisins are mycotoxins produced by *Fusarium* species, principally *Fusarium verticillioides*. They cause equine leukoencephalomalacia, porcine pulmonary edema and have been implicated as a risk factor for human esophageal cancers (International Programme on Chemical Safety, 2000) and neural tube defects (Gelineau-van Waes et al., 2005) in southern Africa and elsewhere. The most commonly occurring and well-characterized fumonisin, fumonisin B_1 (FB_1) is hepatotoxic and nephrotoxic in various species, a liver tumor promoter (Gelderblom et al., 1988; Carlson et al., 2001), hepatocarcinogenic in BD IX male rats (Gelderblom et al., 1991) and female B6C3F1 mice, and nephrocarcinogenic in male F344/N rats (Howard et al., 2001a). Lesions include apoptosis, mitosis and regeneration (Voss et al., 2001) and perturbations in mitotic/apoptotic

Abbreviations: H&E, hematoxylin and eosin; PPAR, peroxisome proliferator-activated receptor; PP, peroxisome proliferator; TNF, tumor necrosis factor; WY, WY-14,643

* Corresponding author at: US-EPA, MD B143, Research Triangle Park, NC 27711, USA. Tel.: +1 919 541 0092; fax: +1 919 541 4201.
E-mail address: corton.chris@epa.gov (Chris Corton).

0300-483X/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.tox.2006.02.012

balance are likely critical for toxigenesis or carcinogenesis in fumonisin-exposed animals (Dragan et al., 2001; Howard et al., 2001b).

FB_1 and other fumonisins inhibit ceramide synthase (Wang et al., 1991; Norred et al., 1992), thereby disrupting sphingolipid metabolism. Disruption results in among other things, inhibition of de novo ceramide biosynthesis, intracellular accumulation of free sphingoid bases, loss of complex sphingolipids, and increased levels of sphingosine breakdown products including sphingoid base-1-phosphates and phosphatidylethanolamine (Riley et al., 2001). The end result is disruption of sphingolipid metabolism and sphingolipid-mediated regulatory functions that might lead to cytotoxicity, apoptosis, cancer promotion, or carcinogenicity. However, the specific molecular events and signaling pathways involved are not well characterized (reviewed by Merrill et al., 2001; Riley et al., 2001).

Peroxisome proliferators (PP) such as WY-14,643 are a large class of structurally dissimilar industrial and pharmaceutical chemicals that were originally identified as inducers of the size and number of peroxisomes in the livers of exposed mice and rats. In rodents, exposure to PP leads to an orchestration of adaptations consisting of hepatocellular hypertrophy and hyperplasia, suppression of apoptosis and transcriptional induction of fatty acid metabolism genes regulated in parallel with peroxisome proliferation (Corton et al., 2000). Chronic exposure to many PP causes an increased incidence of liver tumors in male and female mice and rats (Reddy and Azarnoff, 1980). The effects of PP in the liver are mediated by the nuclear receptor, peroxisome proliferator-activated receptor α (PPARα). PPARα-null mice lack all of the responses of wild-type mice to PP exposure including increases in liver tumors (Lee et al., 1995; Peters et al., 1997).

The cytokine tumor necrosis factor α (TNFα) has been implicated as a modifier of fumonisin toxicity. Mice lacking a functional TNF receptor 1 (p55) or TNF receptor 2 (p75) were protected against the hepatotoxic effects of FB_1 when the mycotoxin was given subcutaneously for 5 consecutive days (Sharma et al., 2000a, 2001). Mice that over-expressed the human TNFα transgene also exhibited less severe hepatotoxicity than wild-type mice (Sharma et al., 2000b), whereas TNFα-null mice given FB_1 using the same dosing scheme exhibited increased apoptosis and cell proliferation compared to wild-type mice (Sharma et al., 2002). These results show that TNFα signaling pathways, while not obligatory for fumonisin toxicity, none-the-less influence the severity of fumonisin-induced hepatotoxicity in mice. The extent to which TNFα might affect fumonisin toxicity in mice when fumonisin is administered via the diet has not been investigated.

Markers of PPARα activation such as peroxisomal β-oxidation and carnitine acyltransferase were not altered in rats fed up to 25 ppm FB_1 for 2 years (Gelderblom et al., 1996), suggesting that hepatotoxicity in rats does not involve a PPARα-dependent mechanism. However, other reports indicate that some effects of fumonisins might be mediated through a PP-like mechanism. In this regard, two markers of PP exposure, CYP4A and acyl-CoA oxidase were increased in rats after FB_1 treatment (Martinez-Larranaga et al., 1996) and Van Veldhoven et al. (2000) found that recombinant mouse PPARα bound some sphingoid bases, including sphingosine in vitro. The latter finding suggests that FB_1-induced hepatotoxicity in rodents might be mediated, at least in part, by PPARα-dependant signaling.

In the investigations described below, the hypothesis that TNFα and PPARα modulate the hepatotoxic responses in mice orally exposed to fumonisins is tested. Specifically, we examined selected indicators of hepatotoxicity in TNFα-null or PPARα-null mice fed diets supplemented with culture material (CM) of the fumonisin-producing fungus, *F. verticillioides*.

2. Materials and methods

2.1. Culture material and diets

Culture material of *F. verticillioides* strain MRC 826, a fumonisin-producing isolate (WFO Marasas, Medical Research Council, Tygerburg, South Africa), was prepared as previously described (Voss et al., 1996), freeze-dried, ground and stored frozen. The CM diets were prepared by mixing the CM (13%, w/w) with control rodent chow (Diet 7012, Teklad, Madison, WI for studies I and II; NIH 07; Zeigler Brothers, Gardners, PA for study III) using a Patterson Kelley V blender with stirring intensifier bar. The CM nominally provided 300–350 ppm FB_1 to the blended diets based on the amount of FB_1 found in the CM by HPLC (Dresden Osborne et al., 2002). Sound, uncontaminated seed corn (Schlessman Superior Seeds, Milan, OH) was substituted for the CM in the control diets.

2.2. Experimental protocols

Three studies were conducted. The experiments conformed to the federal guidelines for the use and care of laboratory animals and were approved by CIIT Institutional Animal Care and Use Committee. The mice received the diets indicated below and deionized (reversed osmosis) water ad libitum. In studies I

and II, osmotic minipumps (Alzet model 2001, 7-day pumps, 1 μL/h, Alza Corporation, Palo Alto, CA) filled with 16 mg/mL 5-bromo-2′-deoxyuridine (BrdU) in phosphate buffered saline were implanted into the mice 1 day before the start of the experiment.

Study I: male wild-type B6,129F2/J or B6,129-Tnf^{tm1gtc1} (TNFα-null mice; Jackson Labs, Bar Harbor, ME) mice ($n = 5$ males/group) 12 (±1.5) weeks of age were fed the control or CM diet for 8 days. Study II: male SV129 wild-type and PPARα-null mice (Lee et al., 1995) 12 ± 1 weeks of age were fed the control or CM diet for 8 days. These groups also received the (gavage) vehicle beginning day 4. A third group was fed the control diet and was administered daily doses of 50 mg/kg/day WY-14,643 (WY) (ChemSyn Science, Lenexa, KS) suspended in 0.2 mL 1% methylcellulose vehicle (Sigma Chemical, St. Louis, MO) beginning on day 4. Study III: male and female SV129 wild-type or PPARα-null mice 12 weeks (±1 week) of age were fed control or CM diets for 5 weeks.

The mice were observed daily and weighed weekly in all studies (also on day 3 in experiments I and II) and immediately before necropsy. The mice were deeply anesthetized with 100 mg/kg sodium pentobarbital and blood was collected by open cardiac puncture. The mice were then euthanized by exsanguination and the livers removed and weighed. A block of the left, anterior right and median liver lobes along with a transverse section of the ileum of each animal were fixed in formalin for 48 h, transferred to 70% ethanol and stained with hematoxylin and eosin (H&E) for microscopic examination (all studies) and for assessment of BrdU incorporation (studies I and II). For BrdU assessment, the liver specimens were further processed by staining with a BrdU-specific antibody (Caltag Laboratories, South San Francisco, CA). It should be noted that the BrdU assay does not take into account polyploidization and binucleation, events known to play a role in responses to PP (Miller et al., 1996; Lalwani et al., 1997). Additional liver samples were collected at necropsy, cut into 0.5-cm cubes, snap-frozen in liquid nitrogen, and stored at approximately −80 °C.

2.3. Evaluation of hepatotoxicity

H&E stained tissue sections were microscopically examined. Evaluations were done without knowledge of the animals' strain or treatment. The specimens were first evaluated to determine the presence of lesions that were consistent with those induced by fumonisins in mice (National Toxicology Program, 2001; Sharma et al., 1997, 2000a). Accordingly, apoptosis, mitotic figures, hepatic anisocytosis, anisonucleosis, cytomegaly and cytoplasmic eosinophilia were considered evidence of fumonisin exposure. The number of apoptotic bodies and mitotic figures (including obvious metaphase or telophase) then counted by visual re-inspection of 10 (studies I and III) to 15 (study II) fields (10× ocular; 20× objective). Apoptotic bodies were identified according to the morphological criteria given by Kerr et al. (1995).

2.4. Quantitation of hepatocyte proliferation

Light microscopy was performed using a Microphot microscope with a Dage CCD color video camera at a magnification of 700×. The liver sections were analyzed using the object recognition system (CHRIS, Sverdrup Medical/Life Sciences Imaging Systems). The labeling index was calculated for each animal by dividing the number of BrdU-labeled hepatocyte nuclei by the total number of hepatocyte nuclei counted (at least 1000), and the results expressed as a percentage.

2.5. Analysis of sphingolipid levels

Liver sphinganine (Sa) and sphingosine (So) concentrations were determined in studies I and II. Sa, So and the C_{20}-Sa internal standard (provided by A.H. Merrill, Jr. and D.C. Liotta, Emory University, Atlanta, GA) were extracted from the tissues using a modification (Riley et al., 1999) of the methods originally described by Merrill et al. (1988). The extracted free sphingoid bases were derivitized with *ortho*-phthalaldehyde and quantified by comparison of the Sa and So peak areas to that of the internal standard by reverse phase high performance liquid chromatography (HPLC). For HPLC, the instrument was fitted with a C18 reverse phase column (3 μm particle size, 4.6 mm × 50 mm column) (Varian Chromatography Associates, Walnut Creek, CA). The sphingoid bases were eluted with 15% glass distilled water in methanol (1 ml/min flow rate). Excitation and detection wavelengths for the fluorescence detector (RF535 Shimadzu Corporation, Kyoto, Japan) were 335 and 440 nm, respectively (details given in Riley et al., 1994).

2.6. Analysis of protein expression

Whole cell extracts were prepared from 100 to 200 mg of liver as described (Wilcke et al., 1995) and stored at approximately −20 °C until used. Protein expression was determined by Western analysis using anti-rat acyl-CoA oxidase (ACO) antibody (a gift from S. Alexson, Huddinge University Hospital, Huddinge, Sweden).

2.7. Statistics

Statistical procedures generally followed the scheme of Weil and Gad (1980). The controls, CM- and WY-14,643-treated groups of each strain were compared using ANOVA followed by the Duncan's Multiple Range Test (parametric data), the Kruskal Wallis Test followed by the Distribution Free Multiple Comparisons (nonparametric data) or Fischer's Least Significant Differences Test (incidence data). Pathology (apoptosis and mitosis) count data were analyzed using the Mann–Whitney *U* test. Comparisons of similarly treated wild-type and PPARα-null groups were done using Student's *t*-test (parametric data), the Wilcoxon Rank Sum Test (nonparametric data), or Fisher's least significant differences test. All tests were two-tailed and significance was judged at $p < 0.05$.

3. Results

3.1. Hepatic effects of CM in wild-type and TNFα-null mice

No statistically significant changes in body weight, liver to body weight ratios or kidney to body weight ratios were found between the control and CM-fed groups in either strain (data not shown). CM treatment elicited an obvious, moderate to marked hepatotoxic response in the TNFα-null group and their livers were easily distinguished during microscopic examinations from those in the other treatment groups. The lesions were consistent with the hepatic effects of fumonisin in mice (National Toxicology Program, 2001; Sharma et al., 1997, 2002, 2003) and included multifocal hepatocyte apoptosis, mitosis, cytomegaly, anisocytosis, anisonucleosis, and cytoplasmic eosinophilia. Lesions found in the wild-type mice fed the CM were less severe and consisted mainly of a few apoptotic hepatocytes scattered in an otherwise largely unaffected parenchyma. The number of apoptotic hepatocytes counted in the CM-fed TNFα-null livers was significantly increased (Fig. 1) compared to the TNFα-null control mice and was more than 20-fold greater than in the wild-type group fed the CM. In the wild-type group fed CM, the number of apoptotic liver cells per mouse was slightly higher than in the wild-type controls (no apoptosis was noted in either WT or TNFα-null controls), but the difference was not significantly significant.

Mitotic figures were less frequently found in the wild-type mice fed CM (group mean = 3.0 ± 3.4 (S.D.)) than in their TNFα-null counterparts (45 ± 19.6). Average number of mitosis per mouse in the control groups averaged 0 (TNFα-null) to 0.4 ± 0.5 (wild-type). Results of hepatocyte proliferation assessments made by counting BrdU-labeled hepatocytes were consistent with the histopathology findings. Wild-type CM-fed mice exhibited a modest increase in the number of labeled hepatocytes (2.5-fold) (Fig. 1B) whereas the CM-fed TNFα-null mice showed a marked 14-fold increase compared to their control group.

The CM had a greater effect on liver Sa (Fig. 2) and Sa/So ratios in the TNFα-null mice compared to wild-type mice. Sa concentrations were increased ca. 4-fold (not statistically significant) and 12-fold (significantly different from its control and the wild-type CM-fed groups) in wild-type and TNFα-null mice after CM treatment, respectively. Sphingosine levels increased significantly, about 2-fold, after CM treatment in both strains. Mean values (±SD) were: wild-type control = 40.4 (±10.1) (pmoles/20 g wet weight), wild-type CM-fed = 79.6 (±11.5), TNFα-null control = 56.2

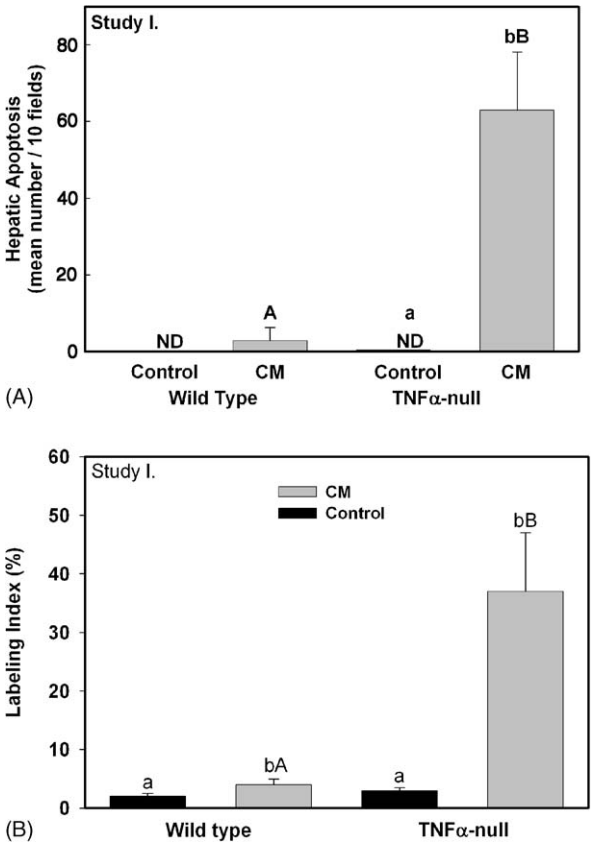

Fig. 1. Liver apoptosis counts (A) and BrdU labeling index (B) in wild-type and TNFα-null mice fed control diets or diets containing *F. verticillioides* CM for 8 days. Values are group means ($n = 5$); error bars denote standard deviation. Values not sharing small case superscripts differ from other groups of the same strain; values having different large case superscripts differ from the corresponding treatment group of the other strain; $p < 0.05$.

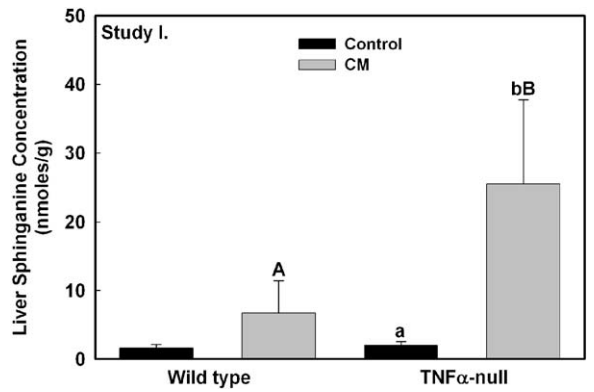

Fig. 2. Liver sphinganine (Sa) concentration of wild-type and TNFα-null mice fed control diet or diet containing *F. verticillioides* CM for 8 days. Values are the group mean ($n = 5$) with error bars denoting standard deviation. Designation of significance is the same as in Fig. 1.

(±9.4), and TNFα-null CM-fed = 94.9 (±11.9). There were no significant differences in sphingosine between the wild-type and corresponding TNFα groups. Thus, largely due to the increased Sa concentrations, there was a 2-fold increase in the liver Sa/So in the wild-type mice fed CM (1.7 versus 0.8 in the wild-type controls; not statistically significant) and a ∼7.7-fold increase in the liver Sa/So in the CM-fed TNFα-null mice (5.5 versus 0.7 in the TNFα-null controls; statistically significant). Together, these findings show that although TNFα was not required for the induction of fumonisin hepatotoxicity by CM in the diet, TNFα gene status had a modulating effect on the level of hepatotoxicity.

3.2. Hepatic effects of CM in wild-type and PPARα-null mice

The role of PPARα in mediating the hepatotoxic effects resulting from short-term (8 days, study II) and longer term (5 weeks, study III) dietary CM exposure was evaluated. In the 8-day study, there were three treatment groups per strain: (1) control diet + control gavage, (2) control diet + WY (50 mg/kg/day) by gavage and (3) CM diet + control gavage. All gavage dosing started on day 4 for 5 days. Liver to body weight ratios were increased in wild-type mice treated with WY (relative liver weight = 5.3%) compared to the control group (4.1%). No statistically significant changes in liver to body weight ratios were found in PPARα-null mice treated with WY or in wild-type or PPARα-null mice fed the CM (data not shown).

WY had no detectable effect on apoptosis in wild-type or PPARα-null mice in the 8-day experiment (Fig. 3A). The wild-type mice fed the CM diet exhibited increased numbers of apoptotic foci although statistical significance was not demonstrated between this group and any other wild-type group or the PPARα-null CM-fed mice due to the large amount of variability (values ranged from 2 to 46 apoptotic foci/mouse) present in this group. The PPARα-null mice exhibited little, if any increase in the number of apoptotic hepatocytes after treatment with CM (0–5 foci per mouse) although the difference between wild-type and PPARα-nulls was not significant. In the five-week experiment (study III), increased numbers of apoptotic foci were found in both wild-type and PPARα-null mice of both sexes (Fig. 3B) and, in each case, the effect was greater in females than males. The principal finding other than apoptosis and mitosis in the CM-exposed mice was minimal to moderate variability of hepatocyte (anisocytosis and cytomegaly) and nucleus (anisonucleosis) which was found in four (wild-type and PPARα-null males) to five (wild-type and PPARα-null females) mice per group. In study III, a few (oncotic) necrotic hepatocytes and focal mixed inflammation infiltrates were also noted. These findings tended to be slightly more severe in females than males and, within each sex, no obvious differences were noted between the wild-type and PPARα-null groups. Although the extent of the apoptotic effect and other findings in the sex/strain/treatment combinations was variable, the results of the two experiments show that PPARα is not required for induction of apoptosis in mouse liver by CM.

In the 8-day study, both wild-type and PPARα-null mice exhibited increased hepatocyte proliferation (labeling index) after exposure to the CM diet (Fig. 3C). The mean labeling index of the wild-type mice exposed to WY was only slightly higher (not statistically significant) than that of the wild-type controls and fewer BrdU-stained nuclei were found in livers from WY-treated PPARα-null mice than in the other PPARα-null groups. Mean mitosis counts also showed considerable variability from animal to animal within each group in study II and, as indicated by the labeling index, were highest in those groups given CM. Group mean values in the wild-type mice were: controls = 0; WY = 0.6 (±0.89; S.D.); CM = 11.8 (±18.8). Those for the PPARα-null groups were: control = 0; WY = 1.0 (±1.0); CM = 4.5 (±5.8). In the five-week experiment, mitosis counts were increased in both sexes and strains by the CM (Table 1). While statistically significant increases in group mean number of mitoses per mouse were shown only in CM-fed females, a statistically significant increase in the number of CM-fed mice having >5 mitosis per liver sample (3× the average mean (1.7) of the four control groups) was found in all sex-strain combinations. Mitosis counts were higher in CM-fed females than in their male counterparts but statistical significance was found only in the wild-type strain. The results of the BrdU labeling studies and mitosis counts during the 8-day and 5-week studies together indicate that hepatocyte proliferation was induced by the CM diet independently of PPARα.

CM exposure for 8 days increased hepatic Sa concentrations (Fig. 4) in both wild-type and PPARα-null mice. WY exposure had no effect on liver Sa concentrations in either strain. No significant differences in liver So were found between the control and CM-fed wild-type mice but So in the PPARα-null mice fed CM was significantly elevated (about 1.5-fold higher). Sphingosine was significantly elevated 3.7-fold by WY in wild-type but there were no changes in PPARα-null mice. As a result of these changes in Sa and So, the liver Sa/So ratio in wild-type mice treated with CM (3.03) was significantly higher (approximately 8.6-fold) than that of the

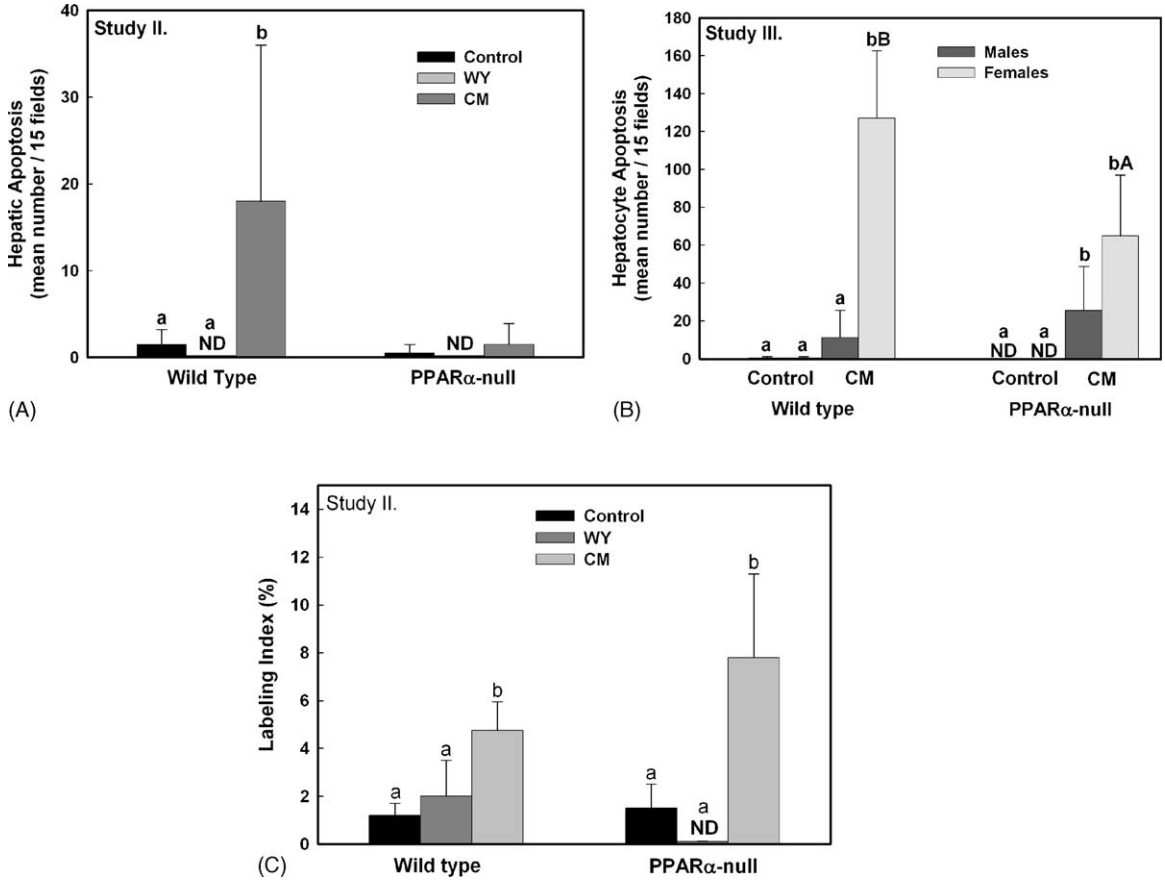

Fig. 3. The effects of a diet containing *F. verticillioides* CM on apoptosis and cell proliferation in wild-type and PPARα-null mice. (A) Liver apoptosis counts in wild-type and PPARα-null mice fed a control diet, a control diet with 4 daily gavage doses of WY-14,643 or a diet containing *F. verticillioides* CM for 8 days. (B) Liver apoptosis counts in wild-type and PPARα-null mice fed a control diet or a diet containing CM for 5 weeks. (C) BrdU labeling index in wild-type and PPARα-null mice fed a control diet, a control diet with 4 daily gavage doses of WY-14,643 or a diet containing *F. verticillioides* CM for 8 days. Values are the group mean ($n = 5$) with error bars denoting standard deviation. Designation of significance is the same as in Fig. 1.

Table 1
Feeding *F. verticillioides* culture material (CM) to wild-type (WT) and PPARα-null (Null) mice for five weeks increased mitosis counts in the liver (see text for explanation of methods)

Strain	Sex	Treatment	Mitoses		
			No. with any	No. with >5	Mean (S.D.)
WT	Male	None (control)	2	0 b	1.4 (2.2)
		CM	5	5 a	9.6 (11.0) B
	Female	None	3	1 b	2.0 (2.5) b
		CM	5	5 a	29 (15) a,A
Null	Male	None (control)	2	0 b	0.4 (2.2)
		CM	4	4 a	20 (26)
	Female	None	4	1 b	3.2 (2.9) b
		CM	5	5 a	44 (23) a

Values not sharing small letters are significantly different from other groups of the same strain; those not sharing large letters differ significantly from those of the corresponding group of the other strain; $p < 0.05$, $n = 5$/group.

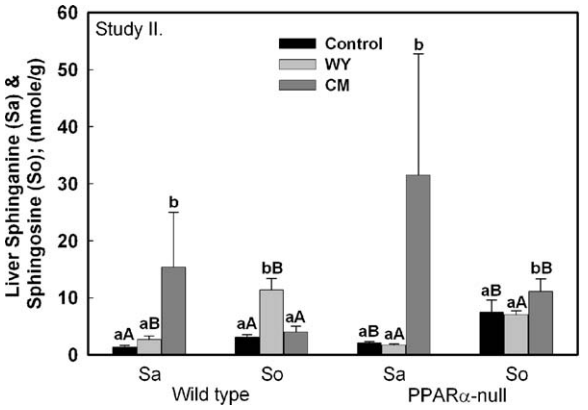

Fig. 4. Liver sphinganine (Sa) concentration of wild-type and PPARα-null mice fed a control diet, a control diet with 4 daily gavage doses of WY-14,643 or a diet containing *F. verticillioides* CM for 8 days. Values are the group mean ($n = 5$) with error bars denoting standard deviation. Designation of significance is the same as in Fig. 1.

control wild-type group (0.31). The Sa/So ratio was also significantly higher (9.8-fold) in the CM treated PPARα-null group then in the PPARα-null controls. WY had no effect on the Sa/So ratio in either strain. Together these data indicate that disruption of sphingolipid metabolism by the fumonisin-containing CM occurred in mice lacking PPARα and that WY increases So levels through a PPARα-dependent mechanism.

3.3. Induction of acyl-CoA oxidase (ACO) by CM

In study I, ACO was modestly induced by CM exposure in both wild-type and TNFα-null mice (Fig. 5). ACO in wild-type mice in study II was also modestly induced by the CM diet and was markedly induced by WY alone (Fig. 5). While WY induction of ACO was completely abolished in the PPARα-null mice, ACO induction by CM was retained in this strain. These results suggest that fumonisins or other compounds in the CM increased ACO protein levels by a PPARα-independent mechanism.

4. Discussion

In this study we tested the hypothesis that TNFα and PPARα are important modulators of toxicity in the mouse liver after exposure to fumonisin. Both fumonisins and PPARα activators can induce TNFα gene and protein levels, increase hepatocyte proliferation and alter apoptosis sensitivity (Corton et al., 2000; Klaunig et al., 2003) and TNFα, like fumonisins, affects sphingolipid metabolism, although by different mechanisms. FB_1 acted as a weak PP in the livers of rats (Martinez-Larranaga et al., 1996) and sphingoid bases, whose concentrations increase in target organs of animals exposed to fumonisins, bind to mouse PPARα (Van Veldhoven et al., 2000).

Disruption of sphingolipid metabolism and hepatotoxicity in mice exposed to fumonisins has been repeatedly correlated (Sharma et al., 1997, 2000a,b, 2002). While TNFα affects sphingolipid metabolism by increasing cell ceramide through activation of sphingomyelinase, the role of TNFα in fumonisin-induced hepatic injury is unclear. Previous in vivo investigations have compared the effects of five consecutive daily doses of 2.25 mg FB_1/kg body weight given by subcutaneous injection, between mice nullizygous for components of

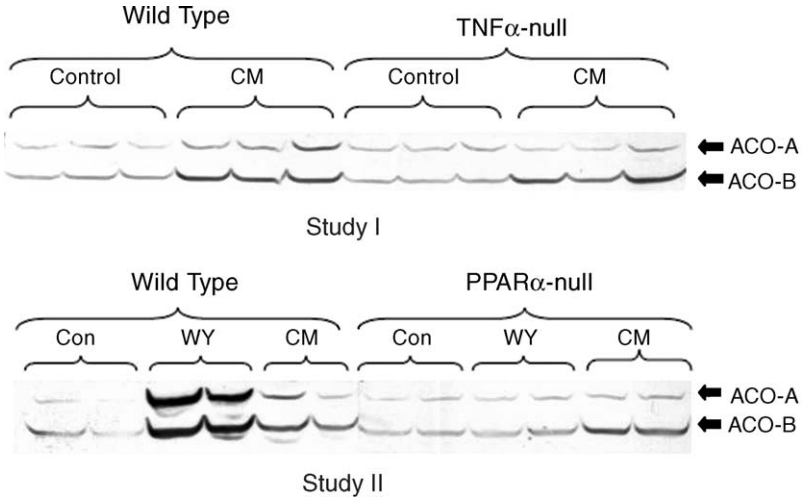

Fig. 5. Acyl-CoA oxidase protein expression in whole cell extracts of liver of wild-type mice and TNFα-null (study I) or PPARα-null (study II) mice. Protein expression was determined by Western analysis using anti-rat acyl-CoA oxidase (ACO) antibody.

TNFα signaling cascades and their wild-type counterparts; the results have been contradictory. Apoptosis, Sa, Sa/So ratios and other measures of fumonisin toxicity were reduced in mice lacking either of the two TNFα receptors, TNFR2 (P75) (Sharma et al., 2000a) or TNFR1 (P55) (Sharma et al., 2001). In contrast, double nullizygous mice lacking both TNFR1 and TNFR2 were not protected against FB_1 and sphingoid base elevations, whereas apoptosis and hepatic lesions were exacerbated in TNFα-null mice (Sharma et al., 2002). In the current investigation, the responses of wild-type and TNFα-null mice were compared under exposure conditions more like those encountered in human exposures, that is, exposure to a complex mixture of fumonisins and other fungal metabolites in the diet. Despite the differences in protocol design and some quantitative differences in liver apoptosis counts (higher in our study) and Sa and Sa/So increases (higher in our study) found in the TNFα-null mice, our results and conclusions were in agreement with the previous report of Sharma et al. (2002). Specifically, TNFα was not required for hepatotoxicity but was a determinant of the level of toxic response to fumonisin exposure.

The high levels of hepatocyte proliferation in CM-treated TNFα-null mice are in contrast to the impaired hepatocyte proliferation observed in TNFR1-null mice after a two-thirds partial hepatectomy (Yamada et al., 1997, 1998) or after exposure to the hepatotoxicant carbon tetrachloride (Yamada and Fausto, 1998). The mechanism by which CM increases cell proliferation is not known in mice; however, oval cell proliferation has been reported in the livers of rats given FB1 (Lemmer et al., 2004). The significant increase in apoptosis in TNFα-null mice is also in contrast to many studies showing that TNFα-dependant pathways are required for apoptosis. Ceramide is a downstream effector of TNFα-induced apoptosis and its concentration in cells is increased through the activity of sphingomyelinases (reviewed in Okazaki et al., 1998; Billis et al., 1998). Our results, and those of earlier studies (Sharma et al., 2002), indicate that this or other TNFα-dependent pathways are not critical for apoptosis following fumonisin exposure. The critical pathways have not yet been definitively identified although Sharma et al. (2003) demonstrated enhanced expression of CD95 (*Fas*)-ligand and other apoptosis signaling molecules in TNFα-null mice exposed to FB_1.

That the apoptotic response in the liver of the PPARα-null mice in our 8-day experiment study (study II), while consistent with the reported effects of fumonisins in mice (National Toxicology Program, 2001; Sharma et al., 1997, 2000a,b, 2001, 2002, 2003), was much less than in the wild-type strain, suggested that PPARα might mediate, at least in part, CM (putatively fumonisin)-induced hepatotoxicity. However, due to the small number of mice per group, the variable morphological response seen among individual mice, and the presence of disrupted sphingolipid metabolism in both groups, further confirmatory studies were undertaken. In the first of these (study III), significantly increased apoptosis was found in both male and female PPARα-null mice fed the CM for 8 weeks. Secondly, in a separate study both pure FB_1 and the CM induced an obvious hepatotoxic response when fed to the wild-type and PPARα-null strains for 8 days and the severity thereof, including liver sphingoid base concentrations, the number of apoptotic foci found per liver, and gene expression were similar in both strains (Voss et al., 2006).

FB_1 can increase markers of PPARα activation including ACO and CYP4A (Martinez-Larranaga et al., 1996). In the present study, the CM diet increased ACO protein levels in both wild-type and PPARα-null mice by a PPARα-independent mechanism which remains to be defined. One possibility is that fatty acyl-CoA moieties usually incorporated into ceramide could accumulate in cells as a consequence of ceramide synthase inhibition and influence other signaling pathways that control ACO expression. The hepatocyte nuclear factor 4α (HNF4α) has been shown to be activated or repressed by fatty acyl-CoA species depending on the length of the fatty acid side chain (Hertz et al., 1998). Palmitoyl-CoA, is both a substrate of ceramide synthase and an activator of HNF4α (Hertz et al., 1998). As this nuclear receptor binds to the ACO peroxisome proliferator response element in the absence of PPARα (reviewed in Corton et al., 2000), it is possible that fumonisin exposure leads to activation of HNF4α and of the ACO gene. It is also possible that compounds other than fumonisins found in the CM activate ACO independently of PPARα. It is noteworthy that, in another 8-day feeding study of similar design (Voss et al., 2006), that hepatotoxicity induced in wild-type and PPARα-null mice by FB_1 as well as a *F. verticillioides* CM (different from that used in the present study) did not detectably increase ACO expression indicating a CM component(s) other then FB_1 was responsible.

PPARα plays a crucial role in lipid metabolism. Activation of PPARα leads to increased levels of batteries of genes that control the transport and metabolic degradation of lipids through β-oxidation in both the mitochondria and peroxisomes. Lipids with structures similar to Sa and So including saturated, monounsaturated and polyunsaturated fatty acids, prostaglandins and leukotrienes activate PPARα directly (Corton et al., 2000). Sphingosine levels were greater in the wild-type

mice dosed with WY and this increase was shown to be PPARα-dependent. Because of the structural similarity between sphingosine, sphinganine and lipid activators of PPARα, we tested the ability of Sa and So to activate PPARα directly in trans-activation assays in HepG2 cells. Our results indicated that they did not activate PPARα directly, most likely, because they lack a key structural feature, a free acid, required for activation (Lapinskas and Corton, unpublished observation). While this provides additional evidence that Sa and So do not exert hepatotoxicity by directly activating PPARα, further studies are needed to determine how PPARα can affect, or be affected by sphingoid bases and whether the increases in sphingosine observed after WY exposure in this study have toxicological relevance.

In summary, these studies provide further evidence that apoptotic and mitotic effects found in the liver of mice after dietary exposure to fumonisins in *F. verticillioides* CM do not depend on the presence of either TNFα or PPARα. The increased severity of hepatic lesions found in TNFα-null mice corroborates previous reports (Sharma et al., 2002) that, while not required, TNFα does modulate fumonisin-induced hepatotoxicity in mice.

Acknowledgements

We thank Dr. Anja Stauber and the CIIT Animal Care and Necropsy and Histology Units for assistance in performing these studies, Dr. Stefan Alexson for the anti-ACO antibody and Drs. Kevin Gaido and Les Recio for critical review of the manuscript. Part of this work has been published in abstract form (Laughter et al., 2001).

References

Billis, W., Fuks, Z., Kolesnick, R., 1998. Signaling in and regulation of ionizing radiation-induced apoptosis in endothelial cells. Rec. Prog. Horm. Res. 53, 85–92.

Carlson, D.B., Williams, D.E., Spitsbergen, J.M., Ross, P.F., Bacon, C.W., Meredith, F.I., Riley, R.T., 2001. Fumonisin B_1 promotes aflatoxin B_1 and *N*-methyl-*N*′-nitro-nitrosoguanidine-initiated liver tumors in rainbow trout. Toxicol. Appl. Pharmacol. 172, 29–36.

Corton, J.C., Anderson, S.P., Stauber, A., 2000. Central role of peroxisome proliferator-activated receptors in the actions of peroxisome proliferators. Ann. Rev. Pharmacol. Toxicol. 40, 491–518.

Dragan, Y.P., Bidlack, W.R., Cohen, S.M., Goldsworthy, T.L., Hard, G.C., Howard, P.C., Riley, R.T., Voss, K.A., 2001. Implications of apoptosis for toxicity, carcinogenicity, and risk assessment: fumonisin B_1 as an example. Toxicol. Sci. 61, 6–17.

Dresden Osborne, C., Pittman Noblet, G., Enongene, E.N., Bacon, C.W., Riley, R.T., Voss, K.A., 1789-98. Host resistance to *Trypanosoma cruzi* infection is enhanced in mice fed *Fusarium verticillioides* (=*F. moniliforme*) culture material containing fumonisins. Food Chem. Toxicol. 40.

Gelineau-van Waes, J., Starr, L., Maddox, J., Aleman, F., Voss, K.A., Wilberding, J., Riley, R.T., 2005. Maternal fumonisin exposure and risk for neural tube defects in an in vivo mouse model. Birth Defects Res. A: Clin. Mol. Teratol. 73, 487–497.

Gelderblom, W.C.A., Jaskieweicz, K., Marasas, W.F.O., Thiel, P.G., Horak, R.M., Vleggaar, R., Kriek, N.J.K., 1806-11. Fumonisins-novel mycotoxins with cancer promoting activity produced by *Fusarium moniliforme*. Appl. Environ. Microbiol. 54.

Gelderblom, W.C.A., Kriek, N.J.K., Marasas, W.F.O., Thiel, P.G., 1991. Toxicity and carcinogenecity of the *Fusarium moniliforme* metabolite fumonisin B_1 in rats. Carcinogenesis 12, 1247–1251.

Gelderblom, W.C., Snyman, S.D., Lebepe-Mazur, S., van der Westhuizen, L., Kriek, N.P., Marasas, W.F., 1996. The cancer-promoting potential of fumonisin B_1 in rat liver using diethylnitrosamine as a cancer initiator. Cancer Lett. 109, 101–108.

Hertz, R., Magenheim, J., Berman, I., Bar-Tana, J., 1998. Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4 alpha. Nature 392, 512–516.

Howard, P.C., Warbritton, A., Voss, K.A., Lorentzen, R.J., Thurman, J.D., Kovach, R.M., Bucci, T.J., 2001a. Compensatory regeneration as a mechanism for renal tubule carcinogenesis of fumonisin B1 in the F344/N/Nctr BR rat. Environ. Health Perspect. 109 (Suppl. 2), 309–314.

Howard, P.C., Eppley, R.M., Stack, M.E., Warbritton, A., Voss, K.A., Lorentzen, R.J., Kovach, R.M., Bucci, T.J., 2001b. Fumonisin B_1 carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. Environ. Health Perspect. 109 (Suppl. 2), 277–282.

International Programme on Chemical Safety, 2000. Fumonisin B_1. Environmental Health Criteria, 219, World Health Organization, Geneva.

Klaunig, J.E., Babich, M.A., Baetcke, K.P., Cook, J.C., Corton, J.C., David, R.M., DeLuca, J.G., Lai, D.Y., McKee, R.H., Peters, J.M., Roberts, R.A., Fenner-Crisp, P.A., 2003. PPARα agonist-induced rodent tumors: modes of action and human relevance. Crit. Rev. Toxicol. 33, 655–780.

Kerr, J.F.R., Gobè, G.C., Winterford, C.M., Harmon, B.V., 1995. Anatomical methods in cell death. In: Schwartz, L.M., Osborne, B.A. (Eds.), Methods in Cell Biology, vol. 26. Academic Press, San Diego, CA, pp. 2–18.

Lalwani, N.D., Dethloff, L.A., Haskins, J.R., Robertson, D.G., de la Iglesia, F.A., 1997. Increased nuclear ploidy, not cell proliferation, is sustained in the peroxisome proliferator-treated rat liver. Toxicol. Pathol. 25, 165–176.

Laughter, A., Dunn, C., Stauber, A.J., Voss, K.A., Riley, R.T., Corton, J.C., 2001. The role of the peroxisome proliferator activated receptor alpha, and tumor necrosis receptor alpha in modulating the effects of fumonisin in mouse liver. Toxicol. Sci. (The Toxicologist) 60, 159 (abstract).

Lee, S.S.-T., Pineau, T., Drago, J., Lee, E.J., Owens, J.W., Kroetz, D.L., Fernandez-Salguero, P.M., Westphal, H., Gonzalez, F.J., 1995. Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. Mol. Cell. Biol. 15, 3012–3022.

Lemmer, E.R., Vessey, C.J., Gelderblom, W.C., Shephard, E.G., Van Schalkwyk, D.J., Van Wijk, R.A., Marasas, W.F., Kirsch, R.E., Hall Pde, L., 2004. Fumonisin B1-induced hepatocellular and cholangiocellular tumors in male Fischer 344 rats: potentiating effects of 2-acetylaminofluorene on oval cell proliferation and neoplastic development in a discontinued feeding study. Carcinogenesis 25, 1257–1264.

Martinez-Larranaga, M.R., Anadon, A., Diaz, M.J., Fernandez, R., Sevil, B., Fernandez-Cruz, M.L., Fernandez, M.C., Martinez, M.A., Anton, R., 1996. Induction of cytochrome P4501A1 and P4504A1 activities and peroxisomal proliferation by fumonisin B_1. Toxicol. Appl. Pharmacol. 141, 185–194.

Merrill Jr., A.H., Wang, E., Mullins, R.E., Jamison, W.C., Nimkar, S., Liotta, D.C., 1988. Quantitation of free sphingosine in liver by high performence liquid chromatography. Anal. Biochem. 171, 373–381.

Merrill Jr., A.H., Sullards, M.C., Wang, E., Voss, K.A., Riley, R.T., 2001. Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. Environ. Health Perspect. 109 (Suppl. 2), 283–289.

Miller, R.T., Shah, R.S., Cattley, R.C., Popp, J.A., 1996. The peroxisome proliferations WY-14,643 and methylclofenapate induce hepatocyte ploidy alterations and ploidy-specific DNA synthesis in F344 rats. Toxicol. Appl. Pharmacol. 138, 317–323.

Norred, W.P., Wang, E., Yoo, H., Riley, R.T., Merrill Jr., A.H., 1992. In vitro toxicology of fumonisins and the mechanistic implications. Mycopathologia 117, 73–78.

National Toxicology Program, 2001. NTP Technical Report on the Toxicology and Carcinogenesis Studies of Fumonisin B_1 (CAS116355-83-0) in F344 Rats and $B6C3F_1$ Mice (feed studies). TR 496, NIH Publication No. 99-3955, Public Health Service, National Institutes of Health, 2001.

Okazaki, T., Kondo, T., Kitano, T., Tashima, M., 1998. Diversity and complexity of ceramide signaling in apoptosis. Cell Signal. 10, 685–692.

Peters, J.M., Cattley, R.C., Gonzalez, F.J., 1997. Role of PPARα in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator WY-14,643. Carcinogenesis 18, 2029–2033.

Reddy, J.K., Azarnoff, D.L., 1980. Hypolipidemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. Nature 283, 397–398.

Riley, R.T., Wang, E., Merrill Jr., A.H., 1994. Liquid chromatographic determination of sphinganine and sphingosine: use of the free sphinganine-to-sphingosine ratio as a biomarker of consumption of fumonisins. J. A. O. A. C. Int. 77, 533–540.

Riley, R.T., Norred, W.P., Wang, E., Merrill Jr., A.H., 1999. Alteration in sphingolipid metabolism bioassay for fumonisin- and ISP-like activity in tissues. Nat. Toxins 7, 407–414.

Riley, R.T., Enongene, E., Voss, K.A., Norred, W.P., Meredith, F.I., Sharma, R.P., Spitsbergen, J., Williams, D.E., Carlson, D.B., Merrill Jr., A.H., 2001. Sphingolipid perturbations as mechanisms for fumonisin carcinogenesis. Environ. Health Perspect. 109 (Suppl.2), 301–308.

Sharma, R.P., Dugyala, R.R., Voss, K.A., 1997. Demonstration of in situ apoptosis in mouse liver and kidney after short-term exposure to fumonisin B_1. J. Comp. Pathol. 117, 371–381.

Sharma, R.P., Bhandari, N., Riley, R.T., Voss, K.A., Meredith, F.I., 2000a. Tolerance to fumonisin toxicity in a mouse strain lacking the P75 tumor necrosis factor receptor. Toxicology 143, 183–194.

Sharma, R.P., Bhandari, N., Tsunoda, M., Riley, R.T., Voss, K.A., 2000b. Fumonisin hepatotoxicity is reduced in mice carrying the human tumor necrosis factor α transgene. Arch. Toxicol. 74, 238–248.

Sharma, R.P., Bhandari, N., He, Q., Riley, R.T., Voss, K.A., 2001. Decreased fumonisin toxicity in mice with a targeted deletion of tumor necrosis factor receptor 1. Toxicology 159, 69–79.

Sharma, R.P., He, Q., Meredith, F.I., Riley, R.T., Voss, K.A., 2002. Paradoxical role of tumor necrosis factor α in fumonisin-induced hepatotoxicity in mice. Toxicology 180, 221–232.

Sharma, R.P., He, Q., Johnson, V.J., Voss, K.A., 2003. Increased expression of CD95-ligand and other apoptotic signaling factors by fumonisin B_1, a hepatotoxic mycotoxin, in livers of mice lacking tumor necrosis factor α. Cytokine 24, 226–236.

Van Veldhoven, P.P., Mannaerts, G.P., Declercq, P., Baes, M., 2000. Do sphingoid bases interact with the peroxisome proliferator activated receptor α (PPAR-α)? Cell Signal. 12, 475–479.

Voss, K.A., Bacon, C.W., Meredith, F.I., Norred, W.P., 1996. Comparative subchronic toxicity studies of nixtamalized and water-extracted *Fusarium moniliforme* culture material. Food Chem. Toxicol. 34, 623–632.

Voss, K.A., Riley, R.T., Norred, W.P., Bacon, C.W., Meredith, F.I., Howard, P.C., Plattner, R.D., Collins, T.F.X., Hansen, D.K., Porter, J.K., 2001. An overview of rodent toxicities: Liver and kidney effects of *Fusarium moniliforme* and fumonisins. Environ. Health Perspect. 109 (Suppl. 2), 259–266.

Voss, K.A., Liu, J., Anderson, S.P., Dunn, C., Miller, J.D., Owen, J.R., Riley, R.T., Bacon, C.W., Corton, J.C., 2006. Toxic effects of fumonisin in mouse liver are independent of the peroxisome proliferator-activated receptor α. Toxicol. Sci. 89, 108–119.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T., Merrill, A.H., 1991. Inhibition of sphingolipid biosynthesis by fumonisins. J. Biol. Chem. 266, 14486–144890.

Weil, C.S., Gad, S.C., 1980. Applications of methods of statistical analysis to efficient repeated-dose toxicologic tests. 2. Methods for analysis of body, liver, and kidney weight data. Toxicol. Appl. Pharmacol. 52, 214–226.

Wilcke, M., Hultenby, K., Alexson, S., 1995. Novel peroxisomal populations in subcellular fractions from the rat liver. J. Biol. Chem. 270, 6949–6958.

Yamada, Y., Fausto, N., 1998. Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1 but not type 2 tumor necrosis factor receptor. Am. J. Pathol. 152, 1577–1589.

Yamada, Y., Kirillova, I., Peschon, J.J., Fausto, N., 1997. Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. Proc. Natl. Acad. Sci. USA 94, 1441–1446.

Yamada, Y., Webber, E.M., Kirillova, I., Peschon, J.J., Fausto, N., 1998. Analysis of liver regeneration in mice lacking type 1 or type 2 tumor necrosis factor receptor: requirement for type 1 but not type 2 receptor. Hepatology 28, 959–970.